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(54) Title: NEUTRALIZING CONFORMATIONAL EPITOPES OF CHICKEN ANEMIA VIRUS

(57) Abstract

The production of the conformational neutralizing epitope of chicken anemia virus is described and compositions for preventing or treating CAV infections, in particular vaccines less pathogenic than CAV itself. All these compositions were shown to synthesize the conformational neutralizing epitope, necessary for the protective immune response against CAV infections. The invention provides recombinant DNA molecules derived from the CAV genome, and recombinant DNA fragments of the CAV genome integrated in the genome of other virus vectors. In particular, these compositions comprise a sub-unit vaccine, a recombinant live virus vaccine and an attenuated vaccine against CAV infections. The invention also provides the production of neutralizing antibodies directed against CAV and diagnostic kits for the detection of CAV.

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Title: Neutralizing conformational epitopes of Chicken anemia virus .

BRIEF DESCRIPTION OF THE INVENTION

The present invention provides the formation and characterization of the neutralizing conformational epitope of the CAV protein VP1, which is required for eliciting a protective immune response in vaccinated animals.

In particular, the production of neutralizing antibodies againt CAV is described. In a diagnostic test, based on these produced neutralizing antibodies, CAV (particles) are detected.

Various vaccine vectors against CAV infections are disclosed. All these vaccines are less pathogenic than CAV. All these vaccine vector are shown to produce the required neutralizing conformational epitope of CAV.

One vector provides a subunit vaccine, based on a recombinant baculovirus expressing both VP1 and VP2 in the same cell. A second type of vaccine is a live virus vector, comprising a Marek's disease vector stably harboring the coding sequences for VP1 and VP2. The third CAV vaccine comprises genetically attenuated CAV strains with a reduced cytopathogenic effect.

BACKGROUND OF THE INVENTION

Chicken anemia virus (CAV) is a small virus of a unique type with a particle diameter of 23 to 25 nm and a genome consisting of a circular single-stranded (minus-strand) DNA (Gelder blom et al., 1989, Noteborn et al., 1991 and Todd et al., 1990). This DNA multiplies in infected cells via a circular double-stranded replicative intermediate, which was recently cloned and fully sequenced. The CAV genome is 2319 nucleotides long (Noteborn and De Boer, 1990). DNA analysis of CAV strains isolated in different continents revealed only minor

differences among the various isolates (Meehan et al., 1992; accession number M81223, Claessens et al., 1991; D10068, Kato et al., 1995; D31965 and Pallister et al., 1994 Soine et al. 1993 and 1994; L14767). Recently, on the basis of the genome structure, CAV has been placed into the novel virus family of Circoviridae. However, CAV is not related to the other members of this virus family consisting of animal single-stranded circular-DNA viruses, such as porcine circovirus and psittacine beak-and-feather-disease virus (Noteborn and Koch, 1995).

The CAV genome contains one evident promoter/enhancer region (Noteborn et al. 1994) which regulates the CAV transcription. The major transcript from the CAV genome is an unspliced polycistronic mRNA of about 2100 nucleotides encoding three proteins of 51.6 kDa (VP1), 24.0 kDa (VP2) and 13.3 kDa (VP3 or apoptin (Noteborn and Koch, 1995, Noteborn et al., 1992 and Phenix et al., 1994). All three predicted CAV proteins are synthesi zed in CAV-infected cells (Noteborn and Koch, 1995). Immunization with (recombinant) VP1 and VP2 synchronously synthesized in the same cells elicits a protective response and can be used as subunit vaccine against chicken infectious anemia (Koch et al., 1995 and Noteborn and Koch, 1994c).

CAV causes clinical and subclinical disease in chickens, and is recognized as an important avian pathogen worldwide (McIlroy et al., 1992 and McNulty et al., 1991). Day-old chicks are especially susceptible to CAV infections. In these animals lethargy, anorexia and anemia are observed from 10 days after inoculation with CAV. After infection mortality may increase to a maximum of 50%. With increasing age the resistance also increases (Jeurissen et al., 1992). The hematocrit values of chicks that had been infected with CAV at an age of 1-3 days are decreased. CAV infections of 1-21 days old chicks result in a depletion of in particular the thymus cortex. However, in older chickens CAV can subclinically multiply.

CAV infection in older chickens can be determined by the occurence of seroconversion.

The depletion of the cortical thymocytes is considered to cause immunodeficiency resulting in enhanced concurrent infections and to vaccination failures (Noteborn and Koch, 1995). The depletion of thymocytes and most likely also of erythroblatoid cells occur via CAV-induced apoptosis (Jeurissen et al., 1992a). The CAV-encoded protein apoptin is the main inducer of this phenomenon (Noteborn et al., 1994).

Maternal antibodies have been found to give an important protection against CAV infection. Vielitz et al. (1991) reported that hens exposed to CAV from large amounts of antibodies, which are passively transferred to the offspring per ovum. These antibodies protect against chicken infectious-anaemia-associated disease. CAV-neutralizing antibodies were detected in yolk of eggs produced by hens that had been inoculated with lysates of cells that had been co-infected with CAV-recombinant baculovirus and produced all three CAV proteins, or mainly VP1 and VP2. Specific clinical signs did not develop in CAV-challenged progeny that hatched from these eggs (Koch et al., 1995).

Vielitz and Landgraf (1988) have developed a vaccine against infectious anaemia, which is based on CAV propagated in chicken embryos. Currently, this is the only commercially available vaccine. The offspring of vaccinated breeders are protected againt infectious anaemia. Vaccination is possible because when maternal immunity vanished, the birds are susceptible to virus infection without developing the disease. Obviously, the use of live vaccines based on non-attenuated virus harbors risks. Experimental infection of 3-week-old chickens resulted in a comprehensive decrease of the functions of the immune system (McConnell et al., 1993, and 1993a) in the absence of disease. In line with this finding, McIlroy (1992) have provided evidence that CAV also causes

considerable economic losses in the absence of disease: the subclinical disease negatively affected feed conversion and average body weight and increased medication, both of which result in a considerable financial burden. So far, non-pathogenic CAV has not been isolated.

The recombinant CAV proteins synthesized by means of the baculovirus expression system can be used as a subunit vaccine. The recombinant CAV proteins VPI and VP2 have been proven to protect chicks by maternal immunity (Koch et al, 1995). Since the baculovirus vector is an insect-specific virus, known to be non-pathogenic for vertebrates, it can be cultured and supplied to chicken without undue risks (Vlak and Keus, 1990).

In general, live-virus vaccines induce a better immune response and are less expensive than sub-unit vaccines. Therefore, knowledge about the immunogenicity of the various CAV proteins can be used to construct attenuated CAV or other recombinant-virus vectors, such as avian herpes viruses (Nakamura et al., 1992, Morgan et al., 1993) or fowlpox virus (Nazerian et al., 1992, Boyle and Heine, 1993). These recombinant viruses should express the CAV protein VP1 and VP2, in addition to their own proteins, and may be applicable as vaccine for both layer breeders and broiler breeders to protect their offspring against infectious anaemia. In addition, such vectors may be used to protect maternally immune broilers against subclinical disease.

DETAILED DESCRIPTION OF THE INVENTION.

The present invention relates to production and analyses of the neutralizing conformational epitope structure of chicken anemia virus (CAV).

Furthermore, the production of neutralizing monoclonal antibodies directed against CAV is disclosed. It is shown that these neutralizing antibodies are

directed against a conformational epitope of CAV protein VP1.

Also a diagnostic test kit, based on these neutralizing antibodies, for the detection of CAV will be described. This invention provides evidence for the mechanism by which the neutralizing antibodies neutralize CAV particles.

For the formation of the neutralizing conformational epitope of VP1, the synthesis of VP2 within the same cell is required. Recombinant baculovirus expressing only VP1 in insect cells only does not react with neutralizing antibodies directed against CAV, but these neutralizing antibodies will react when VP1 and VP2 are synthesized in one cell.

In purified CAV capsids, however, only VP1 is present. This invention discloses that during the synthesis of VP1, and most likely during its complex formation resulting in the CAV capsids, VP2 binds temporarily to VP1. Denaturation of the CAV capsids results in the destruction of the neutralizing epitope, indicating that the neutralizing epitope is an conformational one.

Besides, the invention relates to vaccines and compositions for preventing or treating virus infections in poultry, in particular infections with CAV.

In particular, the invention relates to vaccines that are less pathogenic than the CAV itself but are still capable of producing the neutralizing conformational epitope. Vaccination of chickens with these type of vaccines will lead to the generation of neutralizing antibodies and thus protect the animals and their progeny against CAV infections.

The invention relates to a baculovirus vector which contains separately on its genome the coding sequences for VP1 or VP2. This recombinant baculovirus is able to synthesize VP1 and VP2 in the same cell, resulting in the

formation of the neutralizing conformational epitope of CAV.

The invention also relates to the construction of a Marek's disease virus (MDV) vectors containing CAV sequences encoding VP1, VP2 proteins. In particular, this recombinant MDV vector synthesizes the recombinant CAV proteins in such a way that the neutralizing conformational epitope of VP1 is properly made.

Furthermore, the invention describes the formation of various attenuated CAV strains, which reveals a reduced cytopathogenic effect in chicken T cells in comparison to a a wild-type-derived CAV strain. The attenuated CAV strains were made by introducing point mutations in a cloned CAV DNA genome, in particular a sequence within the promoter/enhancer region has been mutated. The attenuated CAV mutant strains are able to produce the neutralizing conformational epitope.

Processes for the prophylaxis or control of CAV infections, in particular in chickens, and processes for the preparation of recombinant parts of CAV comprising sequences, and processes for the preparation of vaccine are also subjects of the invention.

Thus, reiterating the invention provides a neutralizing antibody or an antibody fragment or derivative thereof reacting with a conformational epitope of viral protein 1 (VP1) of chicken anaemia virus (CAV) recognized by a monoclonal antibody designated as 132-1, 132-2 or 132-3, as produced by the hybridoma's deposited under no.'s xxxxxx, yyyyyy, zzzzzz at the Insitut Pasteur, Paris, France. A workable experiment for determining whether an antibody is an antibody according to the invention is to see if it cross-reacts (or competes) with an antibody from the deposited hybridomas. Fragments and derivatives of antibodies are well known in the art and hardly need further explanation for the person skilled in the art.

The invention also provides a conformational neutralizing epitope of viral protein 1 of chicken anaemia virus recognized by an antibody as disclosed above. The epitope may be part of a larger molecule, it may for instance be bound to a carrier or the epitope may be repeated (at intervals) in a polypeptide chain. Preferably the epitope is part of a larger part of VP1, because such a molecule will have the right conformation more easily.

As described the conformational epitope will only be present on VP1 if it is produced in one cell, preferably from one vector together with VP2. Therefore the invention provides a method for producing a viral protein 1 comprising a conformational epitope as disclosed above, comprising the expression of said viral protein 1 in one cell together with viral protein 2 of said CAV whereby the genetic information encoding VP1 and the genetic information encoding VP2 are separately present on one recombinant vector.

The invention also includes vectors for use in a method just described comprising as two separate coding sequences the genetic information encoding VP1 and the genetic information encoding VP2. Preferably such a vector is based on Marek's disease virus vector so that a vaccine can be produced giving protection against two pathogens.

A very safe and efficient expression system for the viral proteins according to the invention are vectors which are based on a baculo virus vector. As explained before Baculovirus is generally regarded as safe for use in vertebrates since it cannot infect them.

The invention also provides another way of arriving at vaccines or vaccine components which do have the very important neutralizing comformational epitope, but have reduced pathogenicity. (Attenuated viruses for instance.)

This is achieved by providing a method for providing a viral protein 1 comprising a neutralizing conformational epitope according to the invention, comprising expressing at least a functional part of VP1 and VP2 from genes

encoding them, at least one of which genes is under control of a regulatory sequence derived from the CAV sequence upstream of the transcription intitiation site which regulatory sequence is modified to reduce its efficiency. In this way recomnbinant viruses can be produced which do not replicate as fast and therefor are less pathogenic (i.e. attenuated).

Such virus particles are also part of the invention. As disclosed hereinbefore the modification is preferably in the promoter/enhancer region and most preferably the modification is in the 12 base pair insert in the promoter enhancer region.

Recombinant virus particles obtainable by any method according to the invention are also part of the invention, as are nucleic acids for use in any method according to the invention, for instance vectors comprising a gene encoding at least a functional part of VP1 and a gene encoding a functional part of VP2, at least one of the genes being inder control of a regulatory sequence which is modified to reduce its efficiency.

The antibodies and epitopes according to the invention are also useful in a diagnostic test kit for detecting or determining the presence of CAV or antibodies to CAV in a sample

Furthermore vaccines for the treatment or prophylaxis of CAV associated disease comprising an antibody or an epitope according to the invention together with a suitable adjuvans and/or a suitable vehicle for administration are also provided herewith as are vaccines for the treatment or prophylaxis of CAV associated disease comprising recombinant virus particles according to the invention together with a suitable adjuvans and/or a suitable vehicle for administration.

The invention will be explained in more detail on the basis of the following experimental part. This is only for the purpose of illustration and should not be interpreted as a limitation of the scope of protection.

EXPERIMENTAL

Purification of CAV particles to elicit neutralizing monoclonal antibodies against CAV

For the production of neutralizing monoclonal antibodies against CAV, mice were injected with purified CAV particles.

The supernatant of a 1-liter culture of CAV-infected MDCC-MSB1 cells was fourty times concentrated by means of a MILLITAN 300-kDa filter (Millipore, USA). The supernatant was dialyzed against 10mM Tris(ph 8.7)-1mM EDTA (TE) buffer. Subsequently, sodium dodecyl sulphate (SDS) to an end-concentration of 0.5% was added to the CAV-capsid suspension and incubated for 30 minutes at 37°C. Finally, the CAV capsids were pelleted on a 30% sucrose cushion. The pellet containing the CAV capsids was resuspended in 1 ml TE buffer. Mice were twice injected with 100 µl CAV-capsid suspension.

In-vitro neutralization test.

The supernatants of the candidate monoclonal antibodies were diluted 1:2 and then a two-fold dilution series was made. The diluted supernatants were incubated for 1 hour with 104-105 TCID50 CAV-Cux-1 (Von Bülow et al., 1983, Von Bülow, 1985). Approximately one hundred thousand cells of the T cell line MDCC-MSB1 transformed by Marek's disease virus were infected with this mixture of diluted supernatant and virus (Yuasa, 1983, Yuasa et al. 1983).

Immunofluorescence and immunoperoxidase assay

Cells were fixed with 80% acetone and used for immunofluorescence assays with CAV-specific monoclonal antibodies and goat anti-mouse IgC conjugated with fluorescein, as decribed by Noteborn et al. (1990).

Recombinant-Vp1/VP2-MDV-infected CEF were used for immunoperoxidase stai ning with CAV-pecific monoclonal antibodies, as described by Jeurissen et al. (1988).

Enzyme-linked immunosorbens assay (ELISA)

Microtiter wells (Greiner, FRG) were coated with the CAV-specific neutralizing monoclonal antibody 132.1, which was 1:10,000 diluted in 50 mM sodiumbicarbonate pH 9.6. Wash the wells three times with tapped water containing 0.05% Tween 80. Saturate the wells with 100 µul phosphate-buffered saline containing 4% horse serum, 51 gram/liter NaCl, and 0.05% Tween 80 (saturation buffer) for 30 minutes at 37°C. Wash the wells three times with tap water containing 0.05% Tween 80. Next, 50 uµl of non-diluted chicken serum and 50 µul of thirty times concentrated supernatant containing CAV particles, or 50 µul of a lysate of insect cells containing recombinant VP1 and VP2 proteins, were mixed, added per well and incubated for 1 hour at 37°C. Wash the wells three times with tap water containing 0.05% Tween 80. Add per well 100 uµl a 1:2000 fold dilution of a peroxidase-labeled rabbit an ti-mouse immunoglobulin G conjugate in saturation buffer. Incubate for 1 hour at 37°C. Wash again three times with tap water containing 0.05% Tween 80. Add 100 µl of a standard solution of tetramethylbenzidine, sodiumacetate and hydrogenperoxidase to the wells and incubate for 10 minutes at room temperature. The reactions are blocked with 10% H_2SO_4. The various wells are examined at 450 nm, as standard.

Baculovirus and insect cells.

The recombinant baculovirus AcRP23-lacZ (Bishop, 1992) was obtained from Dr. R. Possee, NERC Institute of Virology, Oxford, England, and the genomic DNA was purified as described by Summers and Smith (1987). Spodoptera frugiperda (Sf9) cell were obtained from the American Tissue Culture Collection (no. CRL 1711).

Baculovirus stocks were grown in confluent monolayers and suspension cultures in TC-100 medium (GIBCO/BRL) containing 10% fetal calf serum, as described by Summers and Smith (1987).

Cloning of CAV DNA.

All CAV DNA sequences are originally derived from the plasmid DNA pIc-20H/CAV-EcoRI (Noteborn and De Boer, 1990). All cloning steps with plasmid DNA were in principle carried out according to the methods described by Maniatis et al. (1982).

DNA transformations were carried out in the E.coli strain HB101. All plasmid were multiplied in large culture under agitation, purified on CsCl gradients, and then by filtration over Sephacryl-S500 columns or by filtrations on QIAGEN-chromatography columns.

Construction and selection of recombinant-VP1/VP2 baculovirus.

Recombinant transfer vector pAcVP1/VP2 DNA was transfected with linearized recombinant baculovirus AcRP23-lacZ DNA, in Sf9 cells. After homologous recombinantion baculoviruses were obtained, which had incorporated in the polyhedrin unit instead of the lacZ the two CAV proteins VP1 and VP2 under regulation of the promoter of the p10 or polyhedrin gene, respectively. In first instance, the recombinant CAV viruses were characterized for the absence of beta-galactosidase activity in plaques of baculovirus-infected insect cells. Further the integration of CAV DNA sequences in the baculovirus genome was determined by means of a CAV-specific DNA probe in a hybridization experiment.

Immunoprecipitation assay

Two days after infection, the cells were incubated with Promix label (ICN, USA) and four hours later, the cells were lysed in E1A buffer (50 mM Tris (pH7.5), 0.1%

Triton-X-100, 250 mM NaCl, 50 mM NaF, and 5 mM EDTA) and incubated with monoclonal antibody 111.1 directed against VP2 for 2 hours at 4°C, washed with E1A buffer and separated on a PAA-SDS gel.

Transfection of MDV DNA in CEFs.

For the construction of recombinant-VP1/VP2 MDV, co-transfections with purified recombinant-VP1/VP2 MDV-transfervector and DNA, isolated from chicken embryo fibro blasts (CEF) infected with MDV Rispens isolate (Van Vloten et al. 1972) were carried out according the method described by Graham and Van der Eb (1973).

Transfection of chicken T cells with mutant-CAV genomes.

The various mutated-CAV clones were digested with EcoRI and the EcoRI fragments containing the CAV (mutated) sequences was recircularized and transfected in MDCC-MSB1 cells, by using the DEAE-dextran method as has been described by Noteborn et al. (1991). As control, the whole procedure was in parallel carried out for the pCAV-EcoRI clone.

RESULTS AND DISCUSSION

The production of neutralizing monoclonal antibodies against CAV.

Noteborn and Koch (1994) have described two types of CAV-specific monoclonal antibodies. One type is directed against VP2, while the other is directed against VP3. None of these monoclonal antibodies reveal a CAV-specific neutralizing activity. Even, more important was that none of these monoclonal antibodies was directed against VP1. We assumed that a neutralizing monoclonal antibody might be directed against VP1, for the capsids contain mainly VP1 (Todd et al., 1990). Below, we describe the production of neutralizing antibodies against CAV.

For the production of neutralizing monoclonal antibodies against CAV, mice were injected with purified CAV particles.

As a first screening for (neutralizing) monoclonal antibodies against CAV, microtitre wells were coated with recombinant-VP1/VP2-baculovirus infected insect cells, which co-synthesized both VP1 and VP2 (see below). CAV-specific antisera with a high neutralizing titer reacted at a dilution of 1:1000 specifically with the recombinant VP1 and/or VP2 products (see below). Several different hybridoma cell lines producing monoclonal antibodies, which specifically reacted with recombinant VP1/VP2 products, were obtained.

A CAV-specific serum neutralization test, showed that three of these monoclonal antibodies obtained, had a neutralizing activity against CAV. These three CAV-specific neutralizing monoclonal antibodies were called 132.1; 132.2 and 132.3.(deposited under no's xxxx,yyyy,zzzz at the Institut Pasteur, France.

Microscopy examinations of the neutralizing monoclonal antibodies directed against CAV.

Immunofluorescence showed that the three neutralizing monoclonal antibodies 132.1, 132.2 and 132.3 recognize specific structures in CAV-infected MDCC-MSB1 cells. None of these monoclonal antibodies reacted with uninfected MDCC-MSB1 cells.

Electron-microscopic analysis was carried out with purified CAV particles incubated with neutralising antibodies against CAV (132.1) or with monoclonal antibodies 111.1 (against VP2) or 111.3 (against VP3). Todd et al. (1990) have reported that purified CAV capsids contain onlya 50-kDa protein, which is most likely VP1. The various monoclonal antibodies were detected by immunogold labeling. Only the neutralizing monoclonal antibodies 132.1 were found to bind to CAV particles. Binding of the monoclonal antibody 132.1 to a CAV particle

resulted in its lysis. Furthermore, CAV capsids, which were lysed due to incubation with the neutralising monoclonal antibody 132.1, showed no binding with monoclonal antibo dies directed against VP2 or VP3.

These results reveal the mechanism by which the neutralizing monoclonal antibodies act: They cause the lysis of the virus capsids, by doing so causing non-infectious particles. Furthermore, these data show that purified CAV particles contain (almost) only VP1.

Neutralizing monoclonal antibodies are directed against an conformational epitope on VP1.

Pepscan analysis (Geysen et al., 1984) revealed that none of the 3 neutralizing monoclonal antibodies reacted significantly with one of the 12-mers derived from VP1 or VP2, or VP3. For the sake of brevity only the data obtained wwith monoclonal antibody 132.1 are shown for VP1 in Figure 1, and for VP2 in Figure 2. These results indicate that the neutralizing monoclonal antibodies are directed against a conformational epitope.

These data were confirmed by the following experiments. Purified CAV particles, dotted on a nylon filter under native conditions, still could react with the neutralizing monoclo nal antibody 132.1. However, after boiling in the presence of SDS, the CAV capsid proteins did not bind to monoclonal antibody 132.1.

Immunoprecipitations experiments, carried under native conditions, as described by Noteborn et al. (1994b), with partially purified CAV particles and monoclonal antibody 132.1, 132.2 or 132.3 showed that a protein of about 50 kDa was precipated by these monoclonal antibodies.

It can be concluded that the presented results indicate that the neutralizing monoclo nal antibodies are directed against an conformational epitope of VP1.

Enzyme-linked immunosorbens assay (ELISA) based on a neutralizing antibody against CAV.

We have developed a complex-trapping-blocking (CTB)-ELISA. One can use enriched CAV particles derived from CAV-infected MDCC-MSB-1 cells or recombinant VP1/VP2 proteins synthesized by means of the above described baculovirus system.

Serum from CAV-infected chickens contains antibodies which will block all epitopes on the CAV capsids or recombinant VP1/VP2. This means that the CAV capsid or recombinant VP1/VP2 will not bind to the coated monoclonal antibody 132.1. Negative serum, howe ver, will allow binding of CAV capsids or recombinant VP1/VP2 to the coated 132.1. A signal smaller than 0.5 of the signal detected with a negative control serum will be examined as positive.

The detection level of our CTB-ELISA are titers of 24 to 25 a determined in a serum neutralization test, which is very sensitive. More than 400 sera were analyzed. Comparison to the serum neutralization test revealed that 96.5% of the positive sera within the serum neutrali zation test were positive within the CTB-ELISA, and 98.3% of the negative sera within the serum neutralization test were negative within the CTB-ELISA.

Construction of a recombinant-VP1/VP2 transfer vector.

The coding sequences for the CAV proteins VP1 and VP2 were cloned into the baculovirus transfer vector pACUW51 (cat. no: 21205P), which was commercially obtained from PharMingen, San Diego, USA. This vector is shown in Figure 3 and contains the polyhe drin flanking region, within their midst the baculovirus polyhdrin promoter and the p10 promo ter and for both transcription units, the required 3'-non-coding transcrioptional sequences including the polyadenylation signals. The transfer vector contains prokaryotic sequences for multiplication in bacteria.

The plasmid pET-16b-VP2 (Noteborn et al., dates not published) contains CAV DNA sequences of positions 380 to 1512. This CAV DNA fragment contains the coding region for VP2 flanked by 484 bp 3'-non-coding CAV DNA sequences. 106 bp downstream of the start codon for VP2 the start codon for VP3 is found in another reading frame. The plasmid pET-16b-VP2 was treated with the restriction enzymes NdeI and NheI, and the sticky ends were filled by means of Klenow polymerase. A 0.8 kb CAV DNA fragment was isolated. The plasmid pAcUW51 was linearized with BamH1, the sticky ends filled by means of Klenow polymerase and finally treated with alkaline phosphatase (CIP). The 0.8 kB CAV DNA fragment was ligated at the linearized pAcUW51 DNA. The orientation of VP2 in pAcUW51 was determined by restriction enzyme analysis. This construct was called pUW-VP2.

The plasmid pET-16b-VP1 (Noteborn et al., data not published) contains CAV DNA sequences of positions 853 to 2319. The CAV DNA insertion contains the complete coding region for the protein VP1 flanked by 117 bp 3'-non-coding CAV DNA sequences. The plasmid pET-16b-VP1 was treated with the restriction enzymes NdeI and EcoRI, and the sticky ends were filled by means of Klenow polymerase. A 1.45 kb CAV DNA fragment was isolated. The plasmid pUW-VP2 was linearized by EcoRI, the sticky ends filled by means of Klenow polymerase and finally treated by CIP. The 1.45 kb CAV DNA fragment was ligated at the linearized pUW-VP2. The orientation of VP1 opposite of the p10 promoter unit was determined by restriction-enzyme analysis, and the final construct pAcVP1/VP2 is shown in Figure 3.

Construction of recombinant-VP1/VP2 baculovirus.

The open reading frames encoding VP1 and VP2 were separately cloned into a single baculovirus transfervector, viz pAcUW51. The CAV sequences encoding VP1 are under the regulation of the baculovirus p10 promoter and VP2 under the regulation of the baculovirus

poyhedrin promoter. Transfection of Sf9 insect cells occurred with 'naked' baculovirus DNA and transfervector DNA. After homologous recombinantion baculovirues were obtained which had incorporated the VP1/VP2 expression unit, integrated in the polyhedrin region of the recombinant baculovirus, instead of the lacZ unit. The recombinant baculoviruses were characterized for the absence of beta-galactosidase activity and integration of CAV DNA sequences.

Expression of the CAV proteins VP1 and VP2 in Sf9 cells.

The simultaneous expression of the CAV proteins VP1 and VP2 in Sf9 cells infected with recombinant-VP1/VP2 baculovirus was analyzed by Coomassie-brilliant blue staining and protein labeling with Promix (ICN, USA) or 3H-leucine (Amersham, UK) and PAA-SDS gel electrophoresis.

In the PCT/NL94/00168 it was shown that lysates of insect cells infected with recombi nant-VP1 baculovirus contained a CAV-specific protein of 52 kD and expression of VP2 by recombinant-VP2 baculovirus in infected insect cells produced a major specific product of 30 kDa. Infection of insect cells with recombinant-VP1/VP2 baculovirus resulted in the synthesis of both the CAV-specific proteins of 52 kD and 30 kD. Both CAV-specific products could be detected as radioactively labeled protein band or Coomassie-brilliant blue stained protein band. The latter result indicates that both products are produced in relatively high levels in recombi nant-VP1/VP2-baculovirus-infected insect cells. Sf9 cells infected with a recombinant-lacZ baculovirus did not contain these CAV-specific proteins.

Thus we have obtained evidence that inoculation in hens of crude lysates of recombinant-VP1/VP2-infected Sf9 cells are able to induce neutralizing antibodies directed against CAV.

The role of VP2 for the formation of a conformational neutralizing epitope of VP1.

As reported in PCT/NL94/00168 and by Koch et al. (1995), simultaneous synthesis and not simply mixing of recombinant CAV proteins VP1 and VP2 is required to obtain a neutralizing and protective immune response. These data suggest that VP2 is a non-structural protein that at some stage of infection is required for virus assembly and/or the correct conformation of VP1, which result(s) in the formation of the neutralizing epitope(s). One explanation of the requirement of VP2 might be that it acts as a scaffold protein that is necessary during the assembly of the virion but absent in the final product (Leibowitz and Horwitz, 1975). Examples of scaffold proteins are the IVa2 and 39kDa proteins of adenovirus (D'Halluin et al., 1978; Persson et al., 1979). These proteins act as scaffolds for the formation of the so-called light capsid, but are removed in the next step. VP2 might function in a similar way during the formation of CAV virions. However, at this stage, we cannot entirely exclude that (very) small amounts of VP2 that remained undetected in electroblots of purified CAV preparations (Todd et al., 1990) or in electron microscopic photographs of lysed CAV particles incubated with immunogold-labeled VP2-specific monoclonal antibodies, as described above, associate with VP1 and form conformational neutralizing epitopes. Recently, weak evidence for the presence of VP2 in gradient-purified CAV was reported (Buchholz, 1994).

In the following experiments, evidence is provided that the neutralizing epitope of VP1 is only (optimal) present, when VP2 is simultaneously synthesized. Insect cells were infected with recombinant-CAV baculoviruses expressing VP1, VP2 (see PCT/NL94/00168) or both VP1 plus VP2. The infected Sf9 cells were harvested 3 or 4 days after infection and used for immunofluorescence tests with the CAV-specific neutralizing monoclonal antibody 132.1. The cells containing only the CAV-specific protein VP2 did

not react at all with the monoclonal antibody 132.1. Cells containing only VP1 revealed a very poor immunofluorescence signal after incubation with monoclonal antibody 132.1. However, insect cells infected with recombinant-VP1/VP2 baculovirus expressing both VP1 and VP2 bound very strongly to the neutralising monoclonal 132.1. PAA-SDS gel electrophoresis of in parallel radioactive-labeled lysates of insect cells expressing VP1, VP2 or VP1 plus VP2, revealed that VP1 is expressed at the same level when expressed only or simultaneously with VP2.

VP1 and VP2 interact temporarily with each other.

The neutralizing epitope of VP1 is only formed when VP2 is present, and therefore is a conformationally distinct epitope. This implies that VP1 and VP2 associate with each other during a short time period. By means of immunoprecipitations under very mild conditions, we have examined whether VP1 could associate with VP2. Sf9 insect cells were infected with recombinant baculoviruses, which synthesized VP1, VP2, or VP1 plus VP2.

The results clearly reveal that monoclonal antibody 111.1 precipates VP2 when VP2 is synthesized alone or in the presence of VP1. In the case that besides VP2, VP1 was expres sed also, VP1 co-precipitated to a small extent with VP2. The monoclonal antibody 111.1 did not detectably precipitate VP1, when VP1 was synthesized in the absence of VP2. These data indicate that VP1 and VP2 are (to a relatively small amount) associated to each other. During this association event, VP1 might obtain its conformation resulting in the neutralizing epitope.

Basis for the development of vaccines against CAV infections.

The above presented results show that for the induction of neutralizing antibodies against CAV, VP1 is required which has a specific conformation. In a

baculovirus expression system, this correct VP1 conformation is only possible, when VP1 plus VP2 or VP1 plus VP2 plus VP3 are simultaneously synthesized. Denaturation of VP1 with e.g. sodium-dodecyl-sulphate result also in the destruction of it neutralizing epitope, indicating that the VP1 neutralizing epitope is an conformational one.

The recombinant CAV products, VP1 plus VP2 or VP1 plus VP2 plus VP3, which will be used for vaccination of laying-hens, can be synthesized by means of the baculovirus system. The CAV proteins can also be synthesized by means of other expression systems, such as yeast cells, via (retro)- viral infection or gene amplification (CHO-dhfr system) in mammalian cell systems.

In principle, the expression of fragments of VP1 (in combination with VP2 or VP2 and VP3) may be sufficient for the induction of a protective immune response. The fact that 12-mers of VP1 can not react with neutralizing antibodies against CAV indicates that larger VP1 fragments are needed for getting the correct VP1 conformation to form the neutralizing epitope. However, one should take into account that minor amino-acid mutations or a few amino-acid deletions might not influence the formation of the neutralizing epitope of VP1.

The fact that 2 or 3 proteins encoded by the CAV open reading frames can induce a protective immune respons is also applicable to the development of living virus vectors. The coding sequences for VP1 plus VP2 or VP1 plus VP2 plus VP3 are then cloned into living virus vectors.

It is also possible that one of the CAV proteins, VP1, VP2 or VP3, separately, but then within the context of a living virus vector, is also suitable for the induction of a protective immune response against CAV infections. The expression of fragments of one of the abo ve-mentioned CAV proteins by living virus vectors may be sufficient for the induction of a protective immune response.

The fact that VP3 causes apoptosis in, i.e. chicken mononuclear cells (PCT/NL94/00168; Noteborn et al., 1994a) favors to construct living virus vectors not expres sing VP3. The replication of i.e. Marek_s disease virus might be negatively influenced by VP3-induced apoptosis.

Alternatively, one can produce attenuated CAV expressing VP1 with the required conformation needed for eliciting a neutralizing and protective immune response agnaist CAV infections.

Construction of a recombinant-MDV-VP1/VP2 transfer vector.

The coding sequences for the CAV proteins VP1 and VP2 were cloned into the Marek's disease virus (MDV) transfervector pMD-US10-SV (Koch, unpublished data). The transfervector contains the SV40 early promoter, which are flanked by sequences of the MDV US10 region, and prokaryotic sequences for multiplication in bacteria. A schematic representa tion of the transfer vector is given in Figure 4.

The CAV coding sequences from position nt 368 to nt 2319 were inserted in the pMD-US10-SV transfervector under the regulation of the SV40 ealy promoter. This recombinant transfervector was checked by restriction enzyme digestion and is called pMD-US10-SV-VP1/VP2.

By introducing a point-mutation at position 549 (T was changed into an A) an extra stopcodon has been introduced in the gene encoding VP3 (Noteborn, unpublished data). Therefore, the inserted CAV sequences will only express VP1 and VP2. Indirect-immunofluorescence analysis of chicken embryo fibroblasts (CEF) transfected with the vector pMD-US10-SV-VP1/VP2 proved that VP1 and VP2 were indeed expressed, whereas VP3 was not.

Construction of recombinant-VP1/VP2 MDV.

Using the Ca-phosphate transfection method, recombinant transfer vector pMD-US10-SV-VP1/VP2 DNA was transfected with MDV (Rispens isolate) DNA,in CEF cells.

After homologous recombination, recombinant MDVs were obtained, which had incor porated the CAV coding sequences in the the US10 region (Nakamura et al., 1992). The recombinant-VP1/VP2 MDVs were characterized for the presence of the expression of VP2. By in-parallel plaque purification and immuno-peroxidase analyis using a monoclonal antibody directed against VP2, several 100%-purified recombinant-VP1/VP2 MDV independent strains were obtained.

Characterization of recombinant-VP1/VP2 MDV.

The correct integration of the CAV-DNA sequences in the MDV genome was determined by means of PCR and restriction-enzyme analysis. By subsequent passaging of the various purified recombinant-VP1/VP2 MDVs, it was shown that they remained stable. No recombinants were obtained with (partial) loss of the VP1/VP2 expresssion unit.

The simultaneous expression of the CAV proteins VP1 and VP2 in CEF cells, which were infected with the recombinant-VP1/VP2 MDV, was proven by indirect immunofluorescence. The monoclonal antibodies CVI-CAV-111.1 (111.1), directed against VP2 and CVI-CAV-132.1 (132.1), directed against VP1 were used.

The monoclonal antibody 132.1 is directed against a neutralizing epitope of VP1. The fact that the neutralizing antibody 132.1 can recognize recombinant-MDV expressed VP1, co-synthesized with VP2, implies that the required neutralizing epitope is present on MDV-expressed VP1. Therefore, vaccination of chickens with recombinant-VP1/VP2 MDV will result in the induction of a neutralizing/protective antibody response. Besides the synthesis of the CAV VP1 and VP2 proteins, the recombinant-VP1/VP2 MDV will synthesize the MDV proteins required for the induction of a protective immune response against MDV infections.

Production of attenuated CAV based on the cloned CAV genome pCAV/EcoRI.

The CAV-EcoRI clone is used for the production of viable infectious CAV particles. To that end, the complete CAV insert, the 2,319-bp EcoRI fragment has to be isolated from the bacterial sequences and recircularized by DNA polymerase treatment. Subsequently, e.g. MDCC-MSB1 cells are transfected with the recircularized EcoRI fragments and after several days wild-type CAV will be produced.

One can introduce mutational changes in the CAV sequences of the CAV-EcoRI clone and isolate, recircularize and transfect in MDCC-MSB1 cells. Instead of wild-type CAV, in principle one can produce mutant CAV and if the mutant is less pathogenic, attenuated CAV. The complete strategy for the production of attenuated CAV is shown in Figure 5.

Construction of CAV genomes containing mutated promoter/enhancer regions.

A remarkable feature of the CAV promoter/enhancer region is a sequence of 4 or 5 near-perfect 21-bp repeats, interrupted by an insert of 12-bp (Noteborn et al., 1991). This region is involved in the regulation of the CAV transcription (Noteborn et al., 1994b).

For the production of attenuated CAV, we have introduced mutational changes in the 12-bp insert/direct-repeat region of the CAV-EcoR1 clone (Noteborn et al., 1991). The mutated promoter/enhancer region of the wild-type (wt) and the various CAV mutants are shown in Fig. 6. The Nae-mutant contains no 12-bp/direct-repeat region sequences at all. Instead of these, a NaeI site (5'-GCCGGC-3') has been introduced, which is given as 'N' in figure 6.

All other mutants contain 4 original direct repeats instead of 5, as is the case for the CAV-EcoRI clone. The various CAV mutants, called '6b'p, '12bp', '24bp' contain changed 12-bp inserts. The mutant 'wt in Nae' and of

course the original CAV-EcoRI clone, called 'wt', contain the original 12-bp insert sequences 5'-AAGAGGCGTTCC-3'.

The CAV mutants '6bp', '12bp', '18bp', '24bp' and 'wt in Nae' contain all additional sequences flanking the 12-bp insert/direct-repeat region. At the 5'-site of this region, the linker 5'-GCCCATGT-3', and at the 3'-site the linker 5'-GATCCGCC-3' has been introduced.

Mutated CAV genomes results in the production of attenuated infectious CAV.

To determine whether the mutated CAV genomes could produce live CAV particles, firstly the synthesis of VP3 was examined. At several timepoints after transfection, a part of the various transfected MDCC-MSB1-cell cultures were aceton-fixed and analysed by indirect immunofluorescence using a monoclonal antibody directed against VP3. In parallel, 1 ml of each culture was added to 9 ml of fresh RPMI-medium, which was supplemented with 10% fetal bovine serum.

Approximately 15% and 90% of the cultured MDCC-MSB1 cells, transfected with 'wt' recircularized CAV genome DNA, contained VP3 after 6 days and 11 days (the cells were one time passaged after transfection), respectively.

The CAV mutant genome 'Nae', lacking the complete 12-bp insert/direct-repeat region, was shown not to produce infectious virus particles. Six days after transfection, at most 2% of the cells produced VP3, which is due to transient expression of the 'Nae' DNA. A similar percentage is obtained with expression plasmid pRSV-VP3 (Noteborn et al., 1994b) encoding VP3 only, and which are known not to replicate in eukaryotic cells. After 3 weeks and several passages, no VP3 was present anymore in the Nae-DNA transfected MDCC-MSB1 cell cultures.

At various time-intervals after transfection, the percentage of VP3-positive cells of the MDCC-MSB1 cultures, transfected with the mutant-DNA genomes '6bp', 12bp', 18bp, 24bp and 'wt in Nae' was significantly lower

compared with cultures, transfected with 'wt'-DNA genome. These data imply that the various CAV mutants replicate less fast than the wild-type CAV. Especially, the mutants '6bp' and '18bp' were reduced in their replication rate. Most likely, the mutational changes of the 12-bp insert results in the lower virus spead of the mutated CAV. The results of these experiments are shown in Figure 7.

Subsequently, we have infected fresh MDCC-MSB1 cultures with supernatants of cells transfected with all examined mutated CAV-DNA genomes. All supernatants, except the one derived from 'Nae'-DNA-transfected cells, were proven to contain infectious virus parti cles. For these cultures, infected with the 'mutated-virus' containing supernatants, it could be shown by indirect immunofluorescence, that cells were present containing VP3.

CAV mutants have a reduced cytopathogenic effect.

In parallel with the above described detection of VP3 synthesis, the transfected cells were analysed whether they had become apoptotic. CAV is known to induce apoptosis, 2-3 days after infection. To that end, the cells were stained with propidium iodide, which stains 'normal' DNA strongly, but apoptotic DNA weakly and/or irregularly. Already, 11 days after transfection almost all MDCC-MSB1 cells transfected with 'wt' DNA were shown to be apoptotic, whereas the majority of the cells, transfected with the various mutated CAV-DNA genome were not. The capacity for inducing apoptosis was shown to be mostly reduced for the CAV mutants '6bp' and '18bp'.

DNA analysis of attenuated CAV.

By polymerase-chain reaction and sequence analysis of the 12-bp insert/direct-repeat region and flanking sequences, it was shown that the original mutations were not changed for the various CAV mutants '6bp', '12bp', '18bp', '24bp' and 'wt in Nae'. These results indicate

that the various CAV mutants are stable, at least after culturing in MDCC-MSB1 for about 1 month and that indeed these CAV mutants are viable and causing a reduced cytopathogenic effect in cultured chicken T cells.

Southern blot analysis of DNA isolated from MDCC-MSB1 cultures, infected with the various CAV mutants, revealed that all viable CAV mutants produced all CAV DNAs, as was described for wild-type CAV. The double-stranded replication intermediate and sing le-stranded DNA were detected for both wild-type as mutated CAV.

Attenuated CAV synthesizes the CAV-specific neutralizing epitope.

By indirect-immunofluorescence, using the neutralizing monoclonal antibody CVI-CAV 132.1 and a monoclonal antibody directed against VP2, viz CVI-CAV 111.2 and VP3, viz CVI-CAV-85.1, it was shown that all mutated CAV genomes were able to synthesize the CAV proteins VP1, VP2 and VP3. Even more important is that the neutralizing monoclo nal antibody 132.1 reacted with cells, infected with the various CAV mutants. This finding indicates that the mutated CAVs will produce the required neutralizing CAV-specific epitope, whcih has to be recognized by the immune system of vaccinated chickens as CAV for eliciting a protecvctive immune response.

It can be concluded that based on the CAV clone pCAV-EcoRI, mutated CAV genomes can be made, resulting in the production of viable mutated CAV particles. These CAV mutants replicate in cultured chicken T cells less fast than wild-type CAV. The cytopa thogenic effect caused by these CAV mutants is also reduced compared to wild-type CAV. The CAV mutants are all able to produce all CAV proteins resulting in the required neutrali zing epitope on VP1.

Therefore, these CAV mutants are attenuated versions of CAV in the field.

Description of the Figures

Figure 1 shows the pepscan analysis of the neutralizing monoclonal antibodies of type 132.1 with peptides (12-mers) derived from VP1.

Figure 2 shows the pepscan analysis of the neutralizing monoclonal antibodies of type 132.1 with peptides (12-mers) derived from VP2.

Figure 3 shows the diagrammatic representation of the recombinant transfer pUW-VP1/VP2.

Figure 4 shows the diagrammatic representation of the recombinant transfer vector pMD-US10-SV-VP1/VP2..

Figure 5 shows the strategy for the production of attenuated CAV-mutants, based on the plasmid pCAV/EcoR1 containing 2319 bp of wild-type CAV.

Figure 6 shows the schematic structure of the CAV promoter/enhancer region of the various mutated and wild-type CAV strains.

Figure 7 shows the VP3-expression rates obtained for the various mutant-CAVs and wild-type CAV after tranfection of mutant or wild-type circularized DNA. The VP3-expression rate are given as percentage of VP3-positive MDCC-MSB1 cells.

Figure 8 gives the sequence of CAV.

REFERENCES

1. Boyle, D.B. and Heine, H.G. 1993. Recombinant fowl pox virus vaccines for poultry. Immunology and Cell Biology 71, 391-397.

2. Buchholz, U. Charakterisierung des Hühneranämievirus (CAV mit Hilfe von monoklonal Antikörpern. Dissertation Free University of Berlin, 1994, Journal no 1738, Berlin, Germany.

3. Claessens, J.A.J., Schrier, C.C., Möckett, A.P.A., Jagt, E.H.J.M. and Sondermeijer, P.A.J. 1991. Molecular cloning and sequence analysis of the genome of chicken anemia virus. Journal of General Virology, 72, 2003-2006.

4. D'Halluin, J.C., Martin, G.R., Torpier, G., and Boulanger, P.A. 1978. Adenovirus type 2 assembly analyzed by reversible cross-linking of labile intermediates. Journal of Virology 26, 198-208.

5. Gelderblom, H., Kling, S., Lurz, R., Tischer, I. and Von Bülow, V. 1989. Morphological characterization of chicken anamia agent (CAA). Archives of Virology 109, 115-120.

6. Gheysen, H.M., Barteling, S.J., and Meloen, R. 1985. Small peptides induce antibodies with a sequence and structural requirement for binding antigen comparable to antibodies raised against the native protein. Proceedings of National Academy of Sciences USA 82, 178-182.

7. Graham, F.L. and Van der Eb, A.J. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52, 456-467.

8. Jeurissen, S.H.M., Janse, M.E., Ekino, S., Nieuwenhuis, P., Koch, G., and De Boer, G.F. (1988). Monoclonal antibodies as probe for defining cellular subsets in the bone marrow, thymus, bursa of Fabricius, and spleen of the chicken. Veterinary Immunology and Immunopa thology 19, 225-238.

9. Jeurissen, S.H.M., Janse, M.E., Van Roozelaar, D.J., Koch, G. and De Boer, G.F. 1992. Susceptibility of thymocytes for infection by chicken anemia virus is

related to pre- and hat ching development. Developmental Immunology 2, 123-129.

10. Jeurissen, S.H.M., Wagenaar, F., Pol, J.M.A., Van der Eb, A.J. and Noteborn, M.H.M. (1992a). Chicken anemia virus causes apoptosis of thymocytes after in-vivo infection and of cell lines after in-vitro infection. Journal of Virology 66, 7383-7388.

11. Kato, A., Fujino, F., Nakamura, T., Ishihama, A., and Otaki, Y. 1995. Gene organization of chicken anemia virus. Virology 209, 480-488.

12. Koch, G., Van Roozelaar, D.J., Verschueren, C.A.J., Van der Eb, A.J., and Noteborn, M.H.M. 1995. Immunogenic and protective properties of chicken anemia virus proteins expressed by baculovirus. Vaccine, 13, 763-770.

13. Leibowitz, J., and Horwitz, M.S. 1975. Synthesis and assembly in adenovirus type 5 temperature-sensitive mutants. Virology 93, 198-208.

14. Maniatis, T., Fritsch, E.F., and Sanbrook, J. (1982). Molecular Cloning: A Laboratory Manual. CSHL Press, New York, USA.

15. McConnell, C.D.G., Adair, B.M. and McNulty, M.S. (1993a). Effects of chicken anemia virus on cell-mediated immune function in chickens exposed to the virus by a natural route. Avian Diseases 37, 366-374.

16. McIlroy, S.G., McNulty, M.S., Bruce, D.W., Smyth, J.A., Goodall, E.A. and Alcorn, M.J. (1992). Economic effects of clinical chicken anemia agent infection on profitable broiler production. Avian Diseases 36, 566-574.

17. McNulty, M.S. 1991. Chicken anaemia agent: a review. Avian Pathology 20, 187-203.

18. Meehan, B.M., Todd, D., Creelan, J.L., Earle, J.A.P., Hoey, E.M. and McNulty, M.S. 1992. Characterization of viral DNA from cell infecetd with chicken anaemia agent: sequence analysis of the cloned replicative form and transfection capabilities of cloned genome frag ments. Archives of Virology, 124, 301-319.

19. Morgan, R.W., Gelb, B. Jr, Pope, C.R. and Sondermeijer, P.J.A. (1993). Efficacy of a herpesvirus of turkeys recombinant vaccine containing the fusion gene of Newcastle disease virus: onset of protection and effect of maternal antibodies. Avian Diseases 37, 1026-1032.

20. Nakamura, H., Sakaguchi, M., Hirayama, Y., Miki, N., Yamamoto, M. and Hirai, K. 1992. Protection against Newcastle disease virus by recombinant Marek_s disease virus serotype-1 expressing the fusion protein of Newcastle disease virus. Proceedings World_s Poultry Congress Symposium, Ponsen and Looijen, Wageningen, The Netherlands, Volume I, pp 332-338.

21. Nazerian, K., Lee, L.F., Yanagida, N. and Ogawa, R. (1992). Protection against Marek_s disease by a fowlpox virus recombinant expressing the glycoprotein B of Marek's disease virus. Journal of Virology 66, 1409-1413.

22. Noteborn, M.H.M. en De Boer, G.F. 1991. Patent application USA/no. 030, 335.

23. Noteborn, M.H.M., De Boer, G.F., Kant, A., Koch, G., Bos, J.L., Zantema, A., and Van der Eb, A.J. 1990. Expression of avian leukemia virus env-gp85 in Spodoptera frigiperda cells by use of a baculovirus expression vector. Journal of General Virology 71, 2641-2648.

24. Noteborn, M.H.M., De Boer, G.F., Van Roozelaar, D.J., Karreman, C., Kranenburg, O., Vos, J.G., Jeurissen, S.H.M., Hoeben, R.C., Zantema, A., Koch, G., Van Ormondt, H., and Van der Eb, A.J. 1991. Characterization of cloned chicken anemia virus DNA that contain all elements for the infectious replication cycle. Journal of Virology 65, 3131-3139.

25. Noteborn, M.H.M. and Koch, G. 1995. Chicken anaemia virus infection: molecular basis of pathogenicity. Avian Pathology 24, 11-31.

26. Noteborn, M.H.M. and Koch, G. 1994. PCT/NL94/ 001688. Chicken anemia virus mutants and vaccines and uses based on the viral proteins VP1, VP2 and VP3 or sequences of that virus coding therefore.

27. Noteborn, M.H.M., Kranenburg, O., Zantema, A., Koch, G., De Boer, and Van der Eb, A.J. 1992. Transcription of the chicken anemia virus (CAV) genome and synthesis of its 52-kDa protein. Gene 118, 267-271.

28. Noteborn, M.H.M., Todd, D., Verschueren, C.A.J., De Gauw, H.W.F.M., Curran, W.L., Veldkamp, S., Douglas, A.J., McNulty, M.S., Van der Eb, A.J., and Koch, G. 1994a. A single chicken anemia virus protein induces apoptosis. Journal of Virology 68, 346-371.

29. Noteborn, M.H.M., Veldkamp, S., Verschueren, C.A.J., Van Roozelaar, D.J., Van der Eb, A.J., and Koch, G. 1994. Molecular-biological characterization of chicken anemia virus. In: Virus Diseases of Poultry-New and Evolving Pathogens, 195-212.

30. Noteborn, M.H.M., Verschueren, C.A.J., Zantema, A., Koch, G. and Van der Eb, A.J. 1994b. Identification of the promoter region of chicken anemia virus (CAV) containing a novel enhancer element. Gene 150, 313-318.

31. Pallister, J., Fahey, K.J., and Sheppard, M. 1994. Cloning and equencing of the chicken anemia virus (CAV) ORF-3 gene, and the development of an ELIA for the detection of serum antibody to CAV. Veterinary Microbiology 39, 167-178.

32. Persson, H., Mathisen, B., Philipson, L., and Petterson, V. 1979. A maturation protein in adenovirus morphogenesis. Virology 93, 198-208.

33. Phenix, K.V., Meehan, B.M., Philipson, L. and McNulty, M.S. 1994. Trancriptional analysis and genome expression of chicken anemia virus. Journal of General Virology 75, 905-909.

34. Rispens, B.H., Van Vloten, H., Mastenbroek, N., Maas, H.J.L., and Schat, K.A. 1972. Control of Marek_s disease in the Netherlands. I. Isolation of an avirulent Marek_s disease virus strain (CVI 988) and its use in laboratory vaccination trials. Avian Diseases 16, 108-125.

35. Soine, C., Watson, S.K., Rybicki, E., Lucio, B., Nordgren, R.M., Parrissh, C.R., and Schat, K.A. 1993.

Determination of the detection limit of the polymerase chain reaction for chicken anemia virus. Avian Diseases 37, 467-476.

36. Soine, C., Renshaw, R., O'Connell, P.H., Watson, .K., Lucio, B. and Schat, K.A. 1994. Sequence analysis of cell culture- and non-cell culture-adaptable strains of chicken infectious anaemia virus. In: Proceedings of the International Synposium on IBDV and CAV, Eds. E.F. Kaleta, U. Heffels-Redmann and H. Lange-Herbst. Rauischholzhausen, Germany. pp 364-365.

37. Todd, D., Creelan, J.L., Mackie, D.P., Rixon, F., and McNulty, M.S. 1990. Purificati on and biochemical characterisation of chicken anemia agent. Journal of General Virology 71, 819-823.

38. Vielitz, E. and Landgraf, H. (1988). Anaemia-dermatitis of broilers: field observations on its occurrence, transmission and prevention. Avian Pathology 17, 113-120.

39. Vielitz, E., Conrad, C., Voss. M., Von Bülow, V., Dorn, P., Bachmeier, J. and Lohren, U. (1991). Immunization against chicken anaemia agent (CAA)-a field trial. Deutsche Tierärztliche Wochenschrift 98, 144-147.

40. Vlak, J.M. and Keus, R.J.A. (1990). Baculovirus expression vector system for pro duction of viral vaccines. In: Viral vaccines. Mizrahi, A. (ed.) Wiley-Liss Inc., New-York, USA. pp 91-123.

41. Von Bülow, V., Fuchs, B., and Bertram, M. 1985. Unteruchungen über den Erreger der Infektiösen Anämie bei Hühnerkücken (CAA) in vitro: Vermehrung, Titration, Serumneutralisationstest und indirekter Immunofluorezenstest. Journal of Veterinary Medicine B 32, 679-693.

42. Von Bülow, V., Fuchs, B., Vielitz, B., and Landgraf, H. 1983. Frühsterblichkeitssyn drom bei Küken nach Doppelinfektion mit dem Virus des Marekschen Krankheit (MDV) und einen Anämie-Erreger (CAA). Journal of Veterinary Medicine B 30, 742-750.

43. Yuasa, N. 1983. Propagation and infectivity titration of the Gifu-1 strain of chicken anaemia agent in a cell line (MDCC-MSB1) derived from Marek's disease lymphoma. National Institute Animal Health Quarterly 23, 13-20.

44. Yuasa, N., Taniguchi, T., Goda, M., Shibatanni, M., Imada, T., and Hihara, H. 1983. Isolation of chicken anemia agent with MDCC-MSB1 cells from chickens in the field. National Institute Animal Health Quarterly 23, 78-81.

CLAIMS

1. A neutralizing antibody or an antibody fragment or derivative thereof reacting with a conformational epitope of viral protein 1 (VP1) of chicken anaemia virus (CAV) recognized by a monoclonal antibody designated as 132-1, 132-2 or 132-3, as produced by the hybridoma's deposited under no.'s xxxxxx, yyyyyy, zzzzzz at the Institut Pasteur, Paris, France.

2. A conformational neutralizing epitope of viral protein 1 of chicken anaemia virus recognized by an antibody according to claim 1.

3. A method for producing a viral protein 1 comprising a conformational epitope according to claim 2, comprising the expression of said viral protein 1 in one cell together with viral protein 2 of said CAV whereby the genetic information encoding VP1 and the genetic information encoding VP2 are separately present on one recombinant vector.

4. A vector for use in a method according to claim 3 comprising as two separate coding sequences the genetic information encoding VP1 and the genetic information encoding VP2.

5. A vector according to claim 4 which is based on Marek's disease virus vector.

6. A vector according to claim 4 which is based on a baculo virus vector.

7. A method for providing a viral protein 1 comprising a neutralizing conformational epitope according to claim 1, comprising expressing at least a functional part of VP1 and VP2 from genes encoding them, which genes are under control of a regulatory sequence derived from the CAV sequence upstream of the transcription intitiation site which regulatory sequence is modified to reduce its efficiency.

8. A method according to claim 3 or 7 whereby VP1 is provided in the form of virus particles.

9. A method according to claim 7 whereby the modification is in the promoter/enhancer region.

10. A method according to claim 9 whereby the modification is in the 12 base pair insert in the promoter enhancer region.

11. Recombinant virus particles obtainable by a method according to claim 7 -10.

12. A nucleic acid for use in a method according to claim 7 -10 comprising a gene encoding at least a functional part of VP1 and a gene encoding a functional part of VP2, at least one of the genes being inder control of a regulatory sequence which is modified to reduce its efficiency.

13. A diagnostic test kit for detecting or determining the presence of CAV or antibodies to CAV in a sample using an antbody according to claim 1 and/or an epitope according to claim 2.

14. A vaccine for the treatment or prophylaxis of CAV associated disease comprising an antibody according to claim 1 or an epitope according to claim 2 together with a suitable adjuvans and/or a suitable vehicle for administration.

15 A vaccinefor the treatment or prophylaxis of CAV associated disease comprising recombinant virus particles according to claim 11 together with a suitable adjuvans and/or a suitable vehicle for administration.

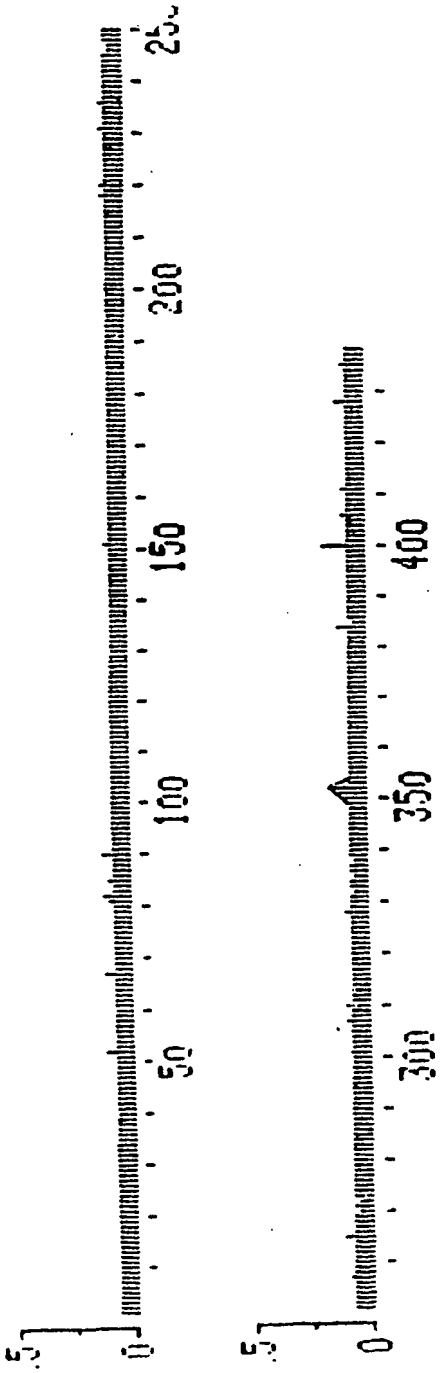

FIG. 1

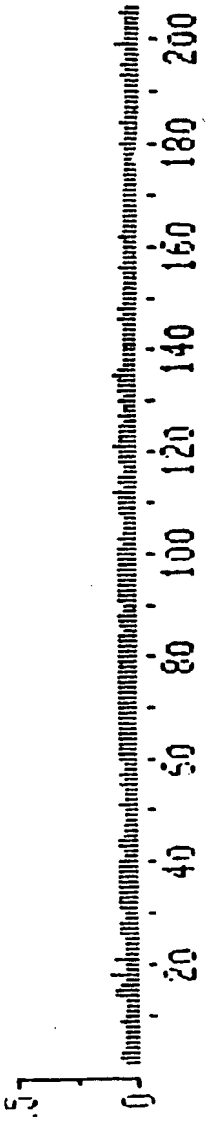

FIG. 2

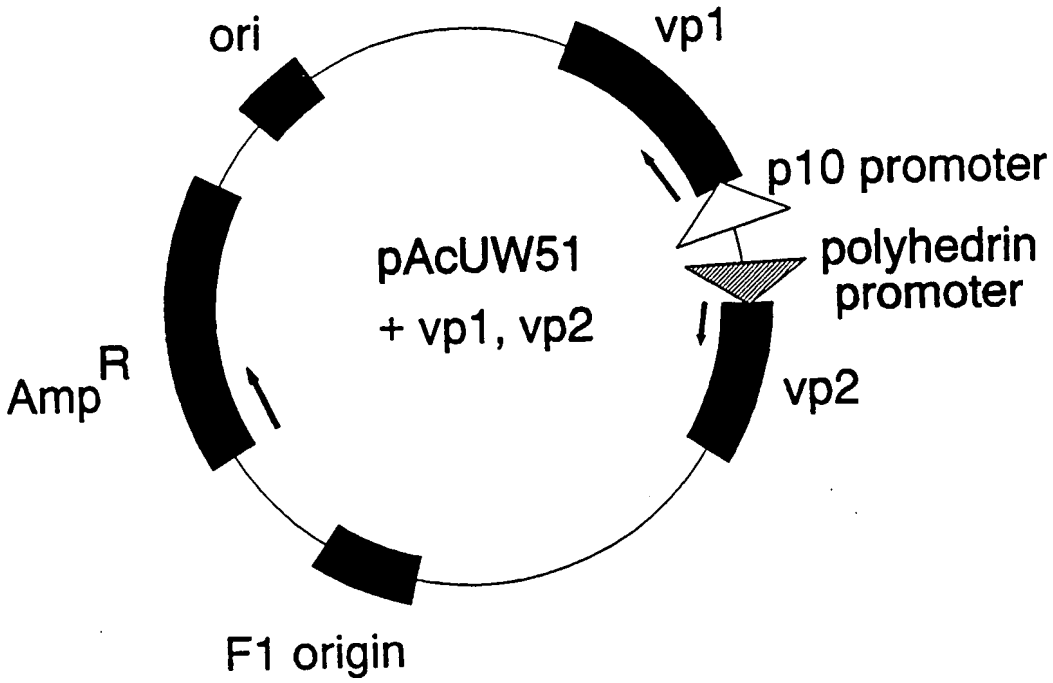
ori
vp1
p10 promoter
polyhedrin promoter
pAcUW51
+ vp1, vp2
vp2
Amp^R
F1 origin

FIG. 3

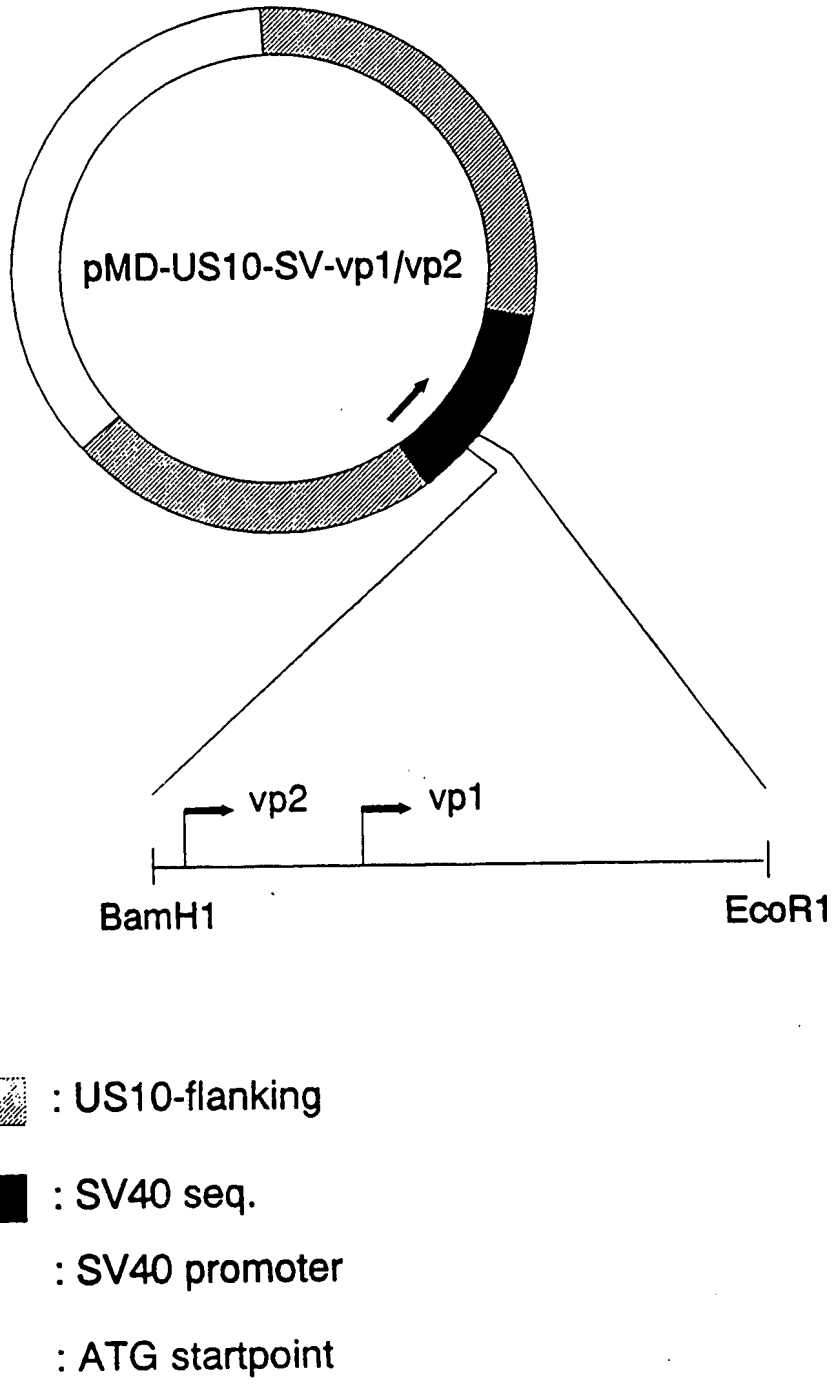
pMD-US10-SV-vp1/vp2
vp2
vp1
BamH1
EcoR1
: US10-flanking
: SV40 seq.
: SV40 promoter
: ATG startpoint

FIG. 4

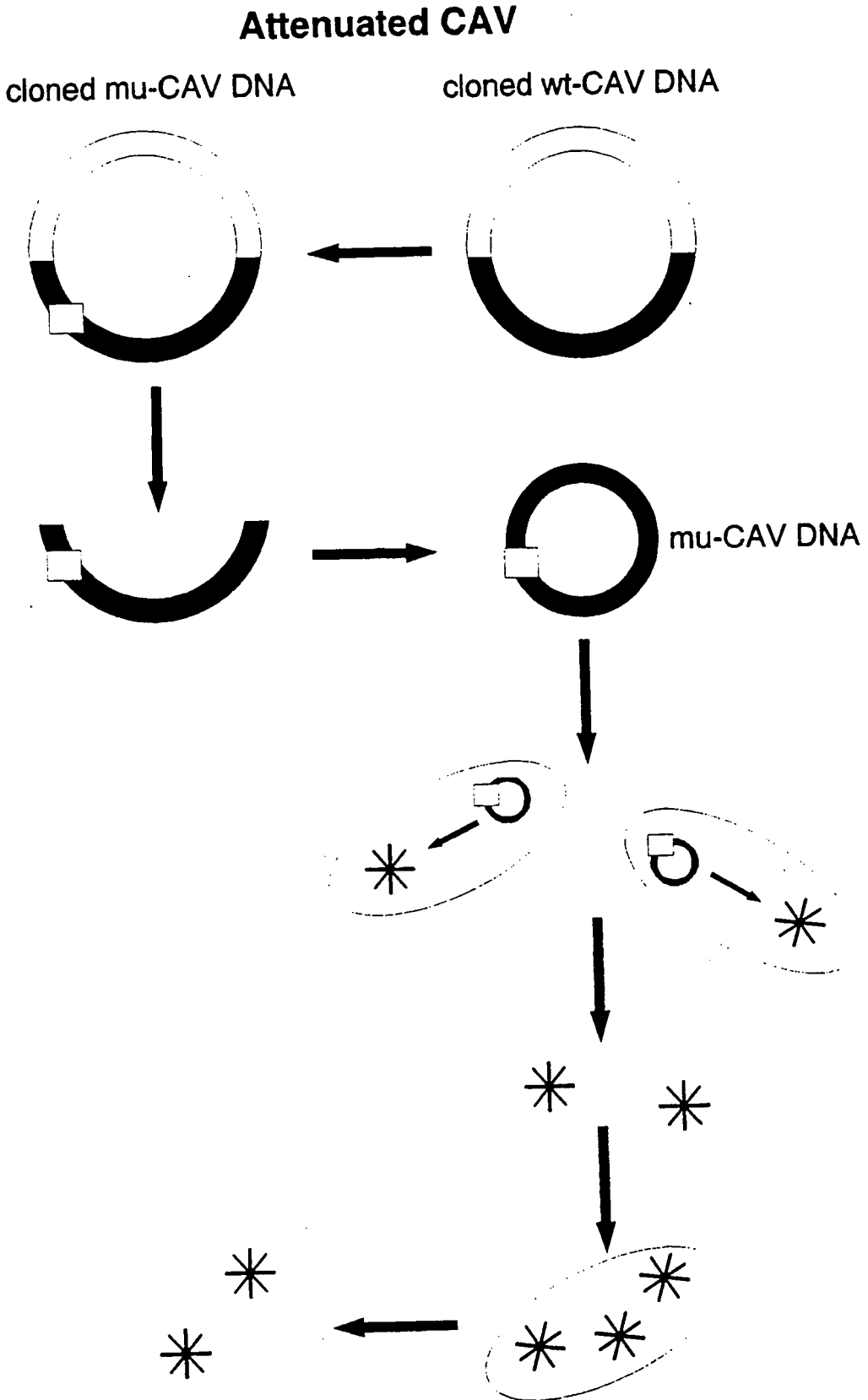

FIG. 5

Promoter/enhancer region of wt en mu CAV strains

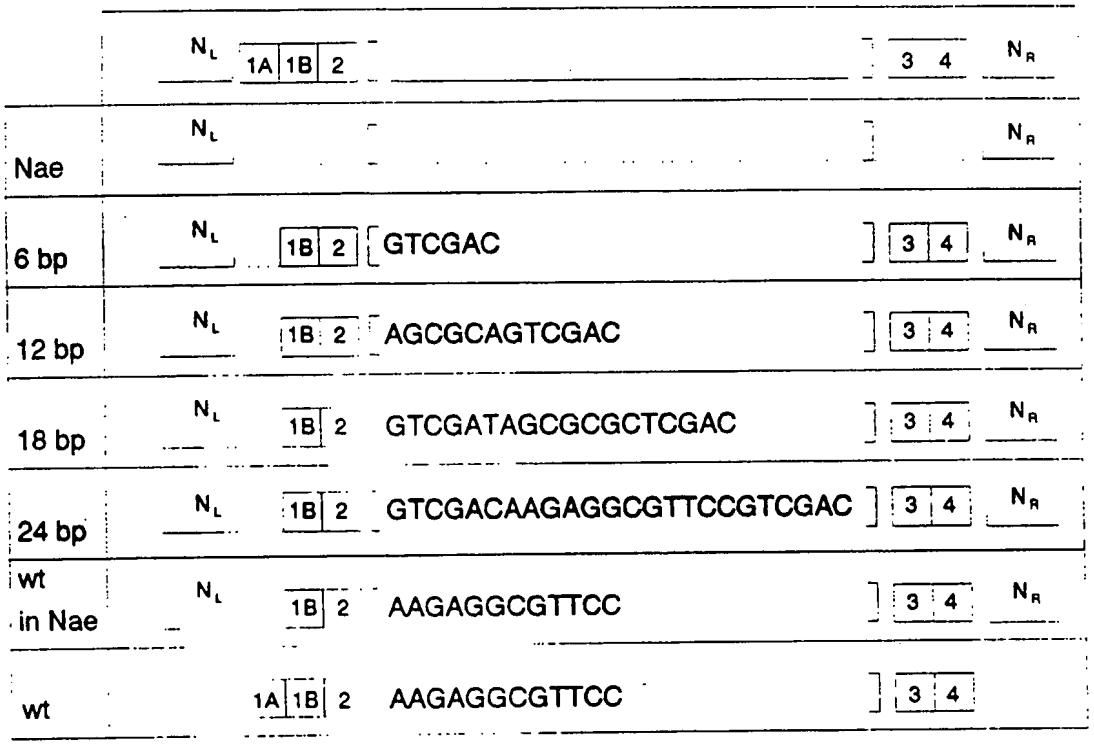

FIG. 6

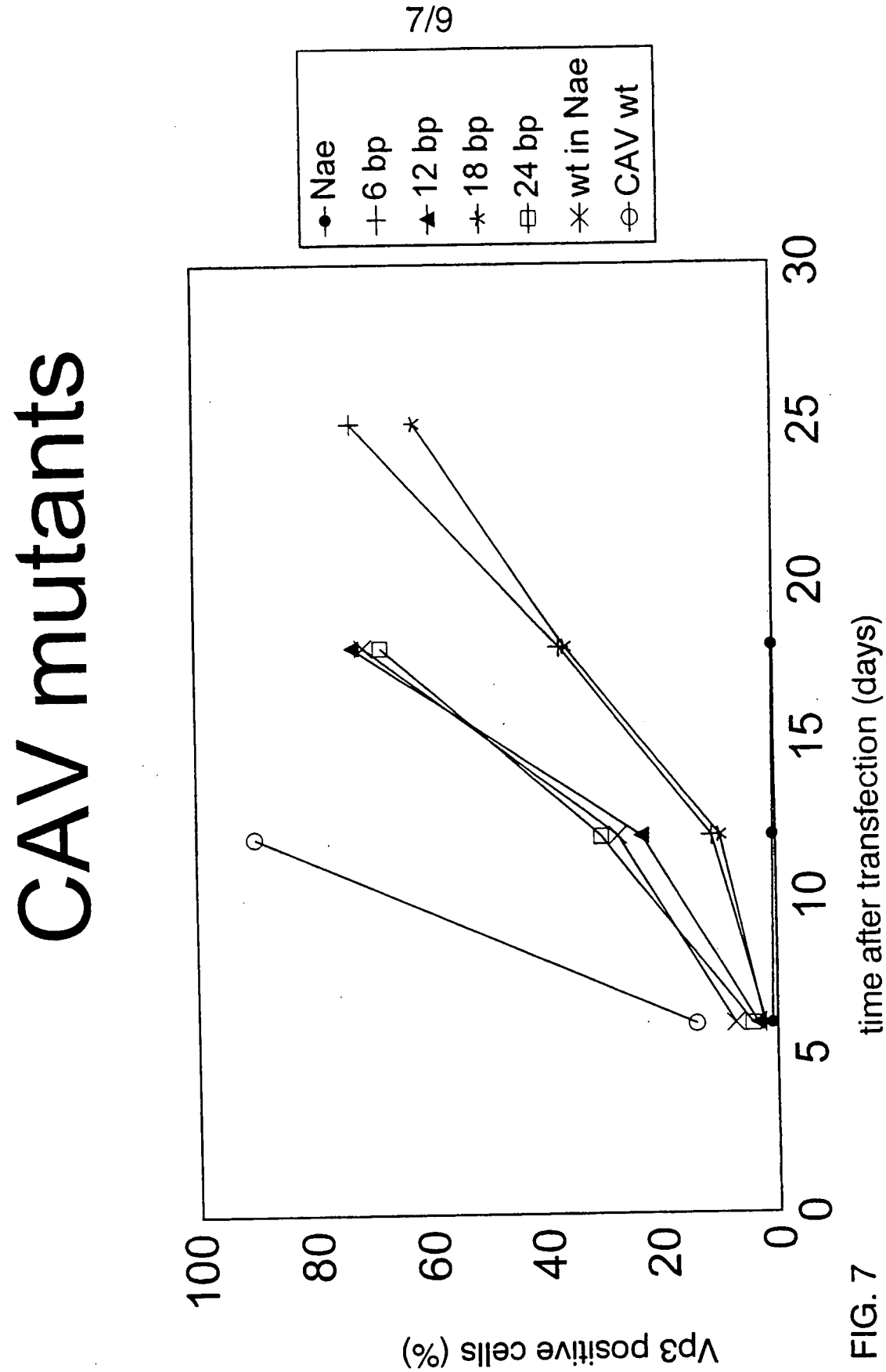
CAV mutants
Nae
6 bp
12 bp
18 bp
24 bp
wt in Nae
CAV wt
Vp3 positive cells (%)
0
20
40
60
80
100
0
5
10
15
20
25
30
time after transfection (days)

FIG. 7

```
                                                                    10
GAATTCCGAG TGGTTACTAT TCCATCACCA TTCTAGCCTG TACACAGAAA GTCAAGATGG ACGAATCGCT CGACTTCGCT CGCGATTCGT CGAAGGCCGG
                                                                    20                  30                  40                  50                  60                  70                  80                  90                  100

                                                                   110
GGGCCGGAGG CCCCCCCGGTC CCCCCCCCTCC AACGAGTGGA GCACGTACAG GGGCGTACGT CATCCGTACA GGGGGGTACG TCATCCGTAC AGGGGGGTAC
                                                                   120                 130                 140                 150                 160                 170                 180                 190                 200

                                                                   210
GTCACAAAGA CGCGTTCCCG TACAGGGGGG TACGTCACGC GTACAGGGGG GTACGTCACA CCCAATCAAA AGCTGCCACG TTGCGAAAGT GACGTTTCGA
                                                                   220                 230                 240                 250                 260                 270                 280                 290                 300

                                                                   310
AAATGGGCGG CGCAAGCCTC TCTATATATT GAGCGCACAT ACCGGTCGGC AGTAGGTATA CGCAAGGCGG TCCGGGTGGA TGCACGGGAA CGGCGCACAA
                                                                   320                 330                 340                 350                 360                 370                 380                 390                 400

                                                                   410
CCGGCCGCTG GGGGCAGTGA ATCGGCGCTT AGCCGAGAGG GGCAACCTGG GCCCAGCGGA CCCGCGCAGG GGCAAGTAAT TTCAAATGAA CGCTCTCCAA
                                                                   420                 430                 440                 450                 460                 470                 480                 490                 500

                                                                   510
GAAGATACTC CACCCGGACC ATCAACGGTG TTCAGGCCAC CAACAAGTTC ACGGCCGTTG GAAACCCCTC ACTGCAGAGA GATCCGGATT GGTATCGCTG
                                                                   520                 530                 540                 550                 560                 570                 580                 590                 600

                                                                   610
GAATTACAAT CACTCTATCG CTGTGTGGCT GCGCGAATGC TCGCGCTCCC ACGCTAAGAT CTGCAACTGC GGACAATTCA GAAAGCACTG GTTTCAAGAA
                                                                   620                 630                 640                 650                 660                 670                 680                 690                 700

                                                                   710
TGTGCCGGAC TTGAGGACCG ATCAACCCAA GCCTCCCTCG AAGAAGCGAT CCTGCGACCC CTCCGAGTAC AGGGTAAGCG AGCTAAAAGA AAGCTTGATT
                                                                   720                 730                 740                 750                 760                 770                 780                 790                 800

                                                                   810
ACCACTACTC CCAGCCGACC CCGAACCGCA AAAAGGCGTA TAAGACTGTA AGATGGCAAG ACGAGCTCGC AGACCGAGAG GCCGATTTTA CTCCTTCAGA
                                                                   820                 830                 840                 850                 860                 870                 880                 890                 900

                                                                   910
AGAGGACGGT GGCACCACCT CAAGCGACTT CGACGAAGAT ATAAATTTCG ACATCGGAGG AGACAGCGGT ATCGTAGACG AGCTTTTAGG AAGCCCTTTC
                                                                   920                 930                 940                 950                 960                 970                 980                 990                 1000

                                                                   1010
ACAACCCCCG CCCCGGTACG TATAGTGTGA GGCTGCCGAA CCCCCAATCT ACTATCACTA TCCGCTTGCA AGGGGTCATC TTTCTCACGG AAGGACTCAT
                                                                   1020                1030                1040                1050                1060                1070                1080                1090                1100

                                                                   1110
TCTGCCTAAA AACAGCACAG CGGCGGGCTA TGCAGACCAC ATGTACGGGG CGAGAGTCGC CAAGATCTCT GTGAACCTGA AAGAGTTCCT GCTAGCCTCA
                                                                   1120                1130                1140                1150                1160                1170                1180                1190                1200
```

FIG. 8(1/2)

```
      1210       1220       1230       1240       1250       1260       1270       1280       1290       1300
ATGAACCTGA CATACGTGAG CAAAATCGGA CGCCCCATCC CCGGTGAGTT GATTGCCCAC GGGTCTAAAT CACAACCCGC GGACAATTGG CCTAATTGCT

      1310       1320       1330       1340       1350       1360       1370       1380       1390       1400
GGCTGCCGCT AGATAATAAC GTGCCCTCCG CTACACCATC GGCATGGTGG AGATGGGCCT TAATGATCAT GCAGCCCACG GACTCTTGCC GGTTCTTTAA

      1410       1420       1430       1440       1450       1460       1470       1480       1490       1500
TCACCCAAAG CAGATGACCC TGCAAGACAT GGGTCCCATG TTTGGGGCCT GGCACCTGTT CCGACACATT GAAACCCGCT TTCAGCTCCT TGCCACTAAG

      1510       1520       1530       1540       1550       1560       1570       1580       1590       1600
AATGAGGGAT CCTTCAGCCC CGTGGCGAGT CTTCTCTCCC AGGGAGAGTA CCTCACGCGT CGGGACGATG TTAAGTACAG CAGCGATCAC CAGAACCGGT

      1610       1620       1630       1640       1650       1660       1670       1680       1690       1700
GGCAAAAAGG CGGACAACCG ATGACGGGGG GCATTGCTTA TGCGACCGGG AAAATCAGAC CCGACGACCA ACACTACCCT GCTATGCCCC CAGACCCCCC

      1710       1720       1730       1740       1750       1760       1770       1780       1790       1800
GATCATCACC GCTACTACAG CGCAAGGCAC GCAAGTCCGC TGCATGAATA GCACGCAAGC TTGGTGGTCA TGGCACACAT ATATGAGCTT TGCAACACTC

      1810       1820       1830       1840       1850       1860       1870       1880       1890       1900
ACAGCACTCG GTGCACAATG GTCTTTTCCT CCAGGGCAAC GTTCAGTTTC TAGACGGTCC TTCAACCACC ACAAGGCGAG AGGACCCGGG GACCCCAAGG

      1910       1920       1930       1940       1950       1960       1970       1980       1990       2000
GCCAGAGATG GCACACGCTG GTGCCGCTCG GCACGGACAC CATCACCGAC AGCTACATGT CAGCACCCGC ATCAGAGCTG GACACTAATT TCTTTACGCT

      2010       2020       2030       2040       2050       2060       2070       2080       2090       2100
TTACGTAGCG CAAGGCACAA ATAAGTCGCA ACAGTACAAG TTCGGCACAG CTACATACGC GCTAAAGGAG CCGGTAATGA AGAGCGATGC ATGGGCACTG

      2110       2120       2130       2140       2150       2160       2170       2180       2190       2200
GTACGCGTCC AGTCGGTCTG GCAGCTGGGT AACAGGCAGA GGCCATACCC ATGGCACGTC AACTGGGCGA ACAGCACCAT CTACTGGGGG ACGCAGCCCT

      2210       2220       2230       2240       2250       2260       2270       2280       2290       2300
CAAAAGGGGG GGGGGCTAAA GCCCCCCCCC CTTAAACCCC CCCCTGGGGG GGATTCCCCC CCAGACCCCC CCTTTATATA GCACTCAATA AAGGCAGAAA

      2310
ATAGATTTAT CCCACTAC
```

FIG. 8(2/2)

INTERNATIONAL SEARCH REPORT

Int onal Application No
PCT/NL 96/00230

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/34 C07K16/08 C07K14/01 C12N15/86 G01N33/569
G01N33/577 A61K39/42 A61K39/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VACCINE, vol. 13, no. 8, June 1995, GUILDFORD, GB, pages 763-770, XP002013395 G. KOCH ET AL.: "Immunogenic and protective properties of chicken anaemia virus proteins expressed by baculovirus." cited in the application see the whole document ---	1-4,6, 13,14
X	WO 95 03414 A (AESCULAAP B.V.) 2 February 1995 cited in the application see examples see claims --- -/--	1-4,6, 13,14

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 September 1996	27.09.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Nooij, F

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INTERNATIONAL SEARCH REPORT

Int onal Application No
PCI/NL 96/00230

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AVIAN PATHOLOGY, vol. 24, no. 1, 1995, HOUGHTON, GB, pages 11-31, XP000577477 M. NOTEBORN ET AL.: "Chicken anaemia virus infection: molecular basis of pathogenicity." cited in the application see abstract see page 17, line 24 - page 19, line 12	1-4,6, 13,14
A	AVIAN DISEASES, vol. 34, no. 2, April 1990, COLLEGE STATION, TX, USA, pages 352-358, XP000577479 M. MCNULTY ET AL.: "Production and preliminary characterization of monoclonal antibodies to chicken anemia agent." see table 1	1
A	GENE, vol. 150, no. 2, 1994, AMSTERDAM, THE NETHERLANDS, pages 313-318, XP002013393 M. NOTEBORN ET AL.: "Identification of the promoter region of chicken anemia virus (CAV) containing a novel enhancer-like element." cited in the application see the whole document	7,9,10
A	JOURNAL OF VIROLOGY, vol. 65, no. 6, June 1991, BALTIMORE, MD, USA, pages 3131-3139, XP002013394 M. NOTEBORN ET AL.: "Characterization of cloned chicken anemia virus DNA that contains all elements for the infectious replication cycle." cited in the application see the whole document	1,2,13
A	WO 92 04446 A (STICHTING CENTRAAL DIERGENEESKUNDIG INSTITUUT ET AL.) 19 March 1992 see claims	1-15
A	EP 0 483 911 A (AKZO N.V.) 6 May 1992 see examples see claims	1-15
A	EP 0 473 210 A (AKZO N.V.) 4 March 1992 see page 4, line 41 - line 50 see claims	4,5

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inv onal Application No
PCT/NL 96/00230

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9503414	02-02-95	NL-A-	9301272	16-02-95
		AU-A-	7547394	20-02-95
		CA-A-	2167578	02-02-95
		ZA-A-	9405275	24-02-95
WO-A-9204446	19-03-92	NL-A-	9002008	01-04-92
		AU-B-	651999	11-08-94
		AU-A-	8658091	30-03-92
		CA-A-	2091181	13-03-92
		EP-A-	0548234	30-06-93
		HU-A-	65974	29-08-94
		JP-T-	6504195	19-05-94
EP-A-483911	06-05-92	AU-A-	8688891	07-05-92
		CA-A-	2054542	01-05-92
		CN-A-	1062168	24-06-92
		JP-A-	5086088	06-04-93
EP-A-473210	04-03-92	AU-B-	645333	13-01-94
		AU-A-	8143791	06-02-92
		CA-A-	2047290	31-01-92
		CN-A-	1060680	29-04-92
		DE-D-	69101683	19-05-94
		DE-T-	69101683	21-07-94
		ES-T-	2056565	01-10-94
		JP-A-	6233682	23-08-94
		US-A-	5231023	27-07-93